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Breast cancers are refractory to TGFß-mediated growth inhibition. We hypothesize that TGFß-resistance can be caused, in principle, by molecular lesions in any of the genes that encode TGFß signaling proteins, that such lesions are likely to occur during the development or progression of human breast cancer, and that they may impact on prognosis or treatment response. We have focused primarily on the the type I (TßR-I) and -II (TßR-II) cell surface receptors for TGFß. This first year of the project, we have generated critical reagents and developed assay systems needed to analyze the structure and function of these two genes in breast cancer specimens:

1. The intron-exon organization of both the TßR-I and -II genes have been resolved. This will allow us to embark on a large scale screening of primary breast carcinoma specimens for the presence of structural alterations of these two genes.
2. We have developed assays using T47D human breast carcinoma cells to test the function of specific TßR-II gene mutants. These will allow us to assess the effects of individual mutations on the ability of TGFß to inhibit cell cycle progression and to induce extracellular matrix-related target genes.

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INTRODUCTION

Transforming Growth Factor-ß (TGFß) is the most potent known inhibitor of cell cycle progression of normal mammary epithelial cells; in addition, it causes cells to deposit increased amounts of extracellular matrix which affects cell-cell and cell-substrate interactions. In general, advanced breast cancers are refractory to TGFß-mediated growth inhibition, while the TGFß they secrete apparently serves to enhance invasion into surrounding structures and perhaps their metastatic potential. The effects of TGFß on cell cycle progression are transduced by two cell surface receptors, TGFß type I (TßR-I) and -II receptors (TßR-II), and relayed from the membrane to the cell nucleus by three recently discovered members of the MAD family of proteins, Smad2, -3, and -4. It is our working hypothesis that TGFß-resistance can, in principle, be caused by molecular lesions in any of these five genes, that such lesions are likely to occur during the development or progression of human breast cancer, and that they may impact on prognosis or treatment response.

This project addresses three of the fundamental research issues raised by the USAMRMC Breast Cancer Research Program. The first question is whether or not molecular lesions of the genes involved in the TGFß signaling pathway contribute to the origin and/or progression of breast cancer. We expect changes in these genes to be relatively late events, perhaps characteristic of metastatic cancer. Secondly, we propose to determine how molecular lesions in the TGFß receptor and/or Smad genes affect receptor function, and how they might play a role in the development and/or progression of breast cancer. Thirdly, we intend to examine the question whether genetic lesions in TGFß receptor and/or Smad genes are able to predict the outcome of patients with breast cancer. Because the anti-tumor effects of anti-estrogens such as tamoxifen are thought to be mediated by the auto- and paracrine induction of TGFß, we wish to test the hypothesis that resistance of hormone-receptor positive cancers to tamoxifen is the result of inactivation of TGFß pathway genes.

BODY

The Statement of Work in our original proposal included the following tasks/timeline:

Task 1. Screening for mutations in TGFß receptor genes in breast cancer

a. Identification of genetic alterations of TGFß-receptor genes in invasive breast cancer specimens. - Months 1-24

b. Identification of genetic alterations of TGFß-receptor gene in sets of pre-invasive, primary invasive and metastatic (lymph node positive) breast cancers in order to determine the stage of tumor development at which these mutations occur. Months 12-36.

Task 2. Determination of the functional consequences of TGFß-receptor mutations

Cloning of TGFß-receptor mutants into mammalian expression vectors and transfection into TGFß-sensitive and -resistant human mammary epithelial cells to determine whether the mutations are dominant or recessive, and correlation of the site of mutations within the molecule with the way they affect the cellular phenotype. - Months 12-36

Task 3. To determine the potential clinical significance of genetic alterations of the TßR-II gene in breast cancer

Test the hypothesis that genetic alterations of TGFß-receptor gene predict for resistance to anti-estrogen therapy in patients with estrogen-receptor positive tumors. Months 36-48.

This report concerns progress achieved on Tasks 1 and 2, which we will describe separately:

Task 1. Screening for mutations in TGFß receptor genes in breast cancer

Our initial studies of genes involved in TGFß signaling focused on the TßR-II gene. Using a chemical mismatch cleavage (CCM) assay, we were the first to identify missense mutations within the TßR-II serine-threonine kinase domain in human head-&-neck cancer cell lines [1]. These findings raised two important questions: (1) Did such structural alterations of the TßR genes also occur in primary tumors (particularly breast cancers) *in vivo*? and (2) How did these mutations in the TßR-II gene affect receptor function?

Before we could embark on a large study of the TßR genes in breast cancer specimens, several stumbling blocks had to be overcome. The first one was the fact that there was no information on the genomic organization of either the TßR-I or -II genes. This type of information is essential for any comprehensive analysis of these genes in cases in which only genomic DNA from primary tissue specimens was available, such as from paraffin-embedded specimens. Furthermore, knowing the intron-exon boundaries would allow us to screen genomic DNA using a simpler and less laborious assay than CCM, based, for example, on single-strand conformation polymorphisms (SSCP). Because only cDNA sequences were available at the time, we were limited to studies of cell lines and/or tissue specimens from which we could extract intact mRNA as template for cDNA synthesis. This represented a serious limitation for primary tumor studies because only a subset of surgically removed primary breast cancers are frozen (~50%), and the quality of extractable mRNA is highly dependent on the rapidity with which the tissue was frozen after surgery. Because we have access to much larger numbers of paraffin-embedded than frozen breast cancer specimens at Yale, we felt that it was important to define the genomic organization of both the human TßR-II and -I genes.

In order to obtain genomic clones that contained the TßR-I gene, a human genomic library cloned into bacterial artificial chromosomes (BAC) [2] was screened using a full-length TßR-I cDNA (ALK-5 [3]) as probe at Genome Systems, Inc. (St. Louis, MO). We obtained three individual BAC clones (BACH-527(A15), -559(G15), -564(C12)) that hybridized with the ALK-5 probe in two sequential screens. In order to confirm that the BAC clones contained TßR-I-specific genomic sequences and to generate a preliminary restriction map, purified BAC DNA was subjected to Southern blot analysis using three different DNA fragments derived from the 5' end, an internal portion and the 3' untranslated region of the TßR-I cDNA, respectively. DNA from two of the three clones (BACH-559 and -564) hybridized strongly with all three ALK-5 probes, even under stringent conditions, indicating that both of these BAC clones encompassed the complete coding sequence as well as the 3' untranslated portion of TßR-I or a related gene. The restriction patterns differed somewhat between clones BACH-559 and -564, suggesting that we might be dealing with two different genes. This was confirmed by PCR analysis. Using two nested sets of oligonucleotide primers specific for TßR-I cDNA sequences and reaction conditions optimized for long-range PCR, we were able to amplify DNA fragments from BACH-559 but not BACH-564. These results indicated that only clone BACH-559 contained the full-length human TßR-I gene sequence.

To determine the location of the intron-exon boundaries within the TßR-I gene, we designed a series of oligonucleotides that matched sequences located at approximately 300 bp intervals along the ALK5 (TßR-I) cDNA, which were then used for direct sequencing of purified BACH-559 DNA. The location of each intron-exon boundary was confirmed by designing a second oligonucleotide primer based on intronic sequences for DNA sequencing in the reverse orientation. As summarized in **Table 1**, the TßR-I gene consists of nine exons, with sizes ranging from 125 to more than 1,000 bp. Exon 1 contains the ATG start codon and extends at least as far upstream as the transcription start site [4]. Most of the extracellular domain of the receptor is encoded by the first two exons. Exon 3 encodes the transmembrane domain, the juxtamembrane

region, as well as the GS-domain. The serine-threonine kinase domain is encoded by exons 4 through 9. Exon 9 encodes the C-terminus of the protein, and extends at least as far as the 3' end of the published ALK5 sequence [3]. Each of the exon-intron junctions follow the GT-AG rule for splice-acceptor and -donor sites. Based on the Southern blot analysis, direct sequencing of BACH-559 DNA, and PCR amplification of individual introns using flanking oligonucleotide primer sets (data not shown), we were able to calculate the molecular sizes of the individual introns (**Table 1**). We estimate that the human TßR-I gene is approximately 31 kb in length.

Table 1. Organization of the TßR-I Gene

Exon	3' Splice acceptor	5' End of exon	Exon size (bp)	3' End of exon	5' Splice donor	Estimated intron size (bp)
1		TGCCTGGCGG	329	GGGGCGACGG	gtgagcggcg	4,900
2	ctttttccag	CGTTACAGTG	246	CCAACTACTG	gtaagttgta	3,200
3	cctttttcag	TAAAGTCATC	231	TCTGGCTCAG	gtaacataat	4,550
4	ttacctttag	GTTTACCATT	231	GACAATAAAG	gtctgtaaca	4,700
5	tgttttacag	ACAATGGTAC	168	GGTACCCAAG	gtaattctat	2,300
6	gattctttag	GAAAGCCAGC	157	GAACAAAAAG	gtatactttt	1,700
7	tttttttag	GTACATGGCC	125	TCCATTGGTG	gtaaattgct	1,100
8	ctgatacag	GAATTCATGA	131	GAGCTGTGAA	gtgagtattt	1,400
9	tttcttgtag	GCCTTGAGAG	≥ 845			

The genomic organization of the segment of the human SKR-2 gene (identical to ActR-IB and ALK-4) that encodes the serine-threonine kinase domain had been reported previously [5]). The intron-exon junctions within the 3' end of the SKR-2/ActR-IB gene match up precisely with those we identified in the human TßR-I gene. Furthermore, there is a high degree of sequence homology between ALK-5 (TßR-I) and other members of the human R-I receptor gene family (ranging from 74% for ActR-IB to 55% for ALK-1) for the region that encodes the juxtamembrane and serine-threonine kinase domains. Thus, it is likely that the region of these genes that encodes the intracellular portion of the respective receptor proteins was derived from a common ancestral gene and that their exon-intron organization is similar. On the other hand, the mouse ActR-IA gene [6] is composed of 10 exons, which only approximately match the locations of the corresponding exons in the human TßR-I and ActR-IB genes, suggesting that this gene has evolved independently of the ActR-IB and TßR-I genes.

Recently two groups of investigators have independently reported the cloning of the human TßR-II gene and its genomic organization [7, 8]. Thus, at this point we have all of the necessary tools to begin screening primary breast carcinoma specimens for structural alterations within the TßR-I and -II genes using an SSCP-based assay. In collaboration with our breast pathologist, Dr. Daryl Carter, we have selected a series of 36 primary stage I and -II breast carcinoma specimens for which both frozen and paraffin-embedded material is available. This panel will allow us to estimate both the frequency of structural alterations of the TßR-I and -II genes in breast cancer, and the levels of mRNA expression of these genes using an RT-PCR-based assay [9].

Task 2. Determination of the functional consequences of TGFß-receptor mutations

As indicated above, we were the first to identify missense mutants (R537P and E526Q) of the TßR-II gene in the TGFß-resistant human head-&-neck SCC cell lines A253 and SqCC/Y1 [1]. Because we anticipate that we will encounter mutations in the TßR-I and -II genes in human breast cancers as well, we needed to develop an experimental strategy that would allow us to precisely determine the consequences of individual mutations for receptor function.

In order to specifically determine which properties of the mutant TßR-II receptors could be

attributed to the E526Q and R537P mutations, we introduced the appropriate nucleotide substitutions into a mouse wild type TßR-II cDNA contained within the p122 expression vector by site-directed mutagenesis [10]. p122 was chosen because it consistently gives high levels of expression of TßR-II protein in mammalian cells [10]. Moreover, the amino acid sequence of the mouse TßR-II is 98% homologous with the human sequence overall, with 100% homology within subdomain XI of the serine-threonine kinase domain where both mutations are located [11]. The properties of these mutant TßR-II cDNAs were analyzed by expressing them in the human breast carcinoma cell line T47D. This cell line was selected because it is refractory TGF-ß1 based on the absence of TßR-II mRNA and protein [12], as indicated by the fact that responsiveness to TGFß can be completely restored by the re-introduction of a wild-type TßRII cDNA [12]. As surrogate markers for the effects of TGFß on cell cycle progression and induction of ECM-associated protein genes we used reporter gene constructs driven by a cyclin A gene promoter (pCAL2) or PAI-1 gene promoter (3TP-Lux), respectively [10, 13].

Transient expression of wild type TßR-II in T47D cells resulted in a greater than 90% reduction in cyclin A promoter activity compared to cells transfected with a control vector, pcDNA3. The repression of the cyclin A promoter occurred independently of the addition of exogenous TGFß1, presumably because T47D cells release significant amounts of active TGFß2 into their culture medium [14]. In contrast, cyclin A promoter activity was repressed by only 20% and 40% in cells transfected with the E526Q and R537P receptor mutants, respectively. Thus, both receptor mutants are deficient in their ability to transduce TGFß's inhibitory effect on cell cycle progression.

Transfection of wild type TßR-II into T47D cells resulted in a 20% increase in PAI-1 gene promoter activity compared to control cells, with an additional 40% increase in response to TGFß1 treatment. In contrast, no induction of the PAI-1 promoter was observed in cells transfected with either the E526Q or the R537P mutant. Thus, these two mutations also abolish the ability of the TßR-II receptor to transduce the effects of TGFß on ECM-associated genes. Both wild type and mutant TßR-II receptors could be detected in transfected T47D cells by immunoprecipitation: Cells transfected with either wild type of mutant TßR-II cDNAs expressed approximately equal levels of TßR-II protein, whereas no endogenous TßR-II was detectable in control transfectants. Moreover, similar amounts of TßR-I receptor protein were detectable in immunoprecipitates of cells transfected with wild type or mutant TßR-II. These results indicate that phenotypic differences observed between the mutant and wild type TßR-II receptors were not due to differences in levels of protein expression nor to differences in the ability of the mutant receptors to form complexes with endogenous TßR-I.

Interestingly, baseline PAI-1 promoter activity was decreased by 55-60% in cells transfected with either the E526Q or the R537P TßR-II mutant compared to control transfectants. One explanation for this observation is that T47D cells express very low levels of wild type TßR-II, which is sufficient to induce PAI-1 promoter activity, and that both mutants (which are expressed at much higher levels) effectively compete for interaction with TßR-I, resulting in a reduction in PAI-1 expression. On the other hand, several studies have suggested that TßR-II is not required for cells to respond to TGFß with the induction of ECM-associated proteins [15, 16]. If this model is correct, our results suggest that both TßR-II mutants interfere with TßR-I receptor function in a dominant-negative fashion. In order to clarify this issue, cells were co-transfected with wild type and mutant TßR-II cDNAs. Neither cyclin A nor PAI-1 promoter activity was affected by co-transfecting a mutant TßR-II with the wild type cDNA. These results indicate that neither mutant is a competitive inhibitor of wild type TßR-II function.

In summary, the human breast carcinoma cell line, T47D, is an ideal target cell line to test the function of TßR-II gene mutants. Both missense mutations in the TßR-II gene that we had previously identified in human head-&-neck cancer cells largely abolish the capability of the TGFß receptor system to transduce TGFß's effects on cell cycle progression as well as induction of

ECM-associated proteins. In addition, our study indicates that these TßR-II mutants inhibit TßR-I function in a dominant-negative fashion.

CONCLUSIONS

1. The intron-exon organization of both the TßR-I and -II genes have been resolved. This allows us to now embark on a large scale screening of primary breast carcinoma specimens for the presence of structural alterations of both genes.

2. We have developed experimental assays using T47D human breast carcinoma cells to test the function of specific TßR-II gene mutants. These assays allow us to assess the effects of individual mutations on the ability of TGFß to inhibit cell cycle progression and to induce extracellular matrix-related target genes.

PUBLICATIONS RESULTING FROM THIS WORK

Reiss, M. ; Barcellos-Hoff, M.H. The role of Transforming Growth Factor-ß in breast cancer-A working hypothesis. Breast Cancer Res. Treatm. In Press:1997.

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APPENDICES

None